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Chemical Identity of Tryptensin with Angiotensin

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1. Tryptensin, a vasopressor substance generated from human plasma protein fraction IV-4 by trypsin, has been isolated and the amino acid composition analysed. 2. The procedures used for the isolation were: (*a*) adsorption of the formed tryptensin on Dowex 50W (X2; NH_4+ form); (*b*) gel filtration through Sephadex G-25; (*c*) cation-exchange chromatography on CM-cellulose; (*d*) anion-exchange chromatography on DEAE-cellulose; (*e*) re-chromatography on CM-cellulose; (*f*) gel filtration on Bio-Gel P-2; (*g*) partition chromatography on high-pressure liquid chromatography. 3. The homogeneity of the isolated tryptensin was confirmed by thin-layer chromatography and thin-layer electrophoresis. 4. The amino acid analysis of the hydrolysate suggested the following proportional composition: Asp, 1; Val, 1; Ile, 1; Tyr, 1; Phe, 1; His, 1; Arg, 1; Pro, 1. This composition is identical with that of human angiotensin.

In a previous paper Arakawa *et al.* (1976) described the formation of tryptensin, an angiotensin-like pressor substance, by trypsin from renin-free human plasma protein. The finding was surprising in contrast with the hitherto well known capability of trypsin to form depressor bradykinin from ox serum, as reported by Elliott *et al.* (1961). It became therefore an urgent problem to clarify the entire system, but, above all, chemical identification of tryptensin was the most urgent, and this is dealt with in the present paper. Fraction IV-4 of human plasma protein was used as substrate. Endogenous renin activity contained in fraction IV-4 (Arakawa *et al.*, 1968) was inactivated by alkali treatment (Arakawa *et al.*, 1975) before use. After incubation of the substrate with trypsin, the formed tryptensin was, by combinations of several chromatographic steps, purified to homogeneity and the amino acid composition was determined.

Experimental

Materials

Human plasma protein fraction IV-4 was obtained from Midori-Juji (Osaka, Japan), synthetic [Asp^1,Ile^5]proangiotensin and [Asp^1,Ile^5]-angiotensin were purchased from the Protein Research Foundation (Osaka, Japan), crystalline bovine trypsin (3000 NF units) was from Miles Laboratory (Slough, Bucks., U.K.), and 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one was from Seikagaku Kogyo (Tokyo, Japan). Dowex 50W (H^+ form; X2; 100–200 mesh) was from Dow Chemicals (Richmond, CA, U.S.A.). CM-52 CM-cellulose and DE-52 DEAE-cellulose were from Whatman (Clifton, NJ, U.S.A.), Sephadex G-25 (medium) was from Pharmacia (Uppsala, Sweden), Bio-Gel P-2 (100–200 mesh) was from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and Avicel-SF cellulose thin-layer plates (10cm × 10cm) were from Funakoshi Chemicals (Tokyo, Japan). The high-pressure-liquid-chromatography apparatus was from Waters Associates (model ALC/GPC 204), and the packing material was μ-Bondapak alkylphenyl. The column dimensions were 4mm (internal diam.) × 30cm.

Methods

Bioassay. Pressor bioassay was done by rat blood pressure as described before (Arakawa *et al.*, 1965, 1967*a*,*b*).

1-Chloro-4-phenyl-3-L-tosylamidobutan-2-one treatment of trypsin. Trypsin samples were treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one by the method of Kostka & Carpenter (1964).

Specific activity. The ratio of pressor activity to A_{280} of samples from each purification step were calculated and used as a measure of specific activity.

Protein and peptide determination. Absorbance was determined by a model 100-300 Hitachi photoelectric spectrophotometer, either at 280nm, when directly read, or 750nm when Lowry's procedure (Lowry *et al.*, 1951) was applied.

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Thin-layer chromatography. Homogeneity of the sample was checked by t.l.c. on Avicel-SF cellulose thin-layer plates (10cm × 10cm). It was developed by the ascending technique with the solvent isopentanol/pyridine/water (7:7:6, by vol.), and located with 0.2% ninhydrin solution in butanol satured with water. It was also checked by cellulose thin-layer electrophoresis on Avicel-SF cellulose thin-layer plates (10cm × 10cm) with pyridine/acetic acid/water (1:10:89, by vol.) of pH3.6. Voltage (400V) was applied for 1h and it was also located by ninhydrin. A portion (25μg) of the isolated sample was hydrolysed by 0.5ml of 6M-HCl in a sealed ampoule with N_2 gas at 110±1°C for 20h. HCl was removed at 50°C under reduced pressure and evaporated to dryness. The hydrolysed sample was analysed by a model 835 Hitachi high-speed amino-acid analyser.

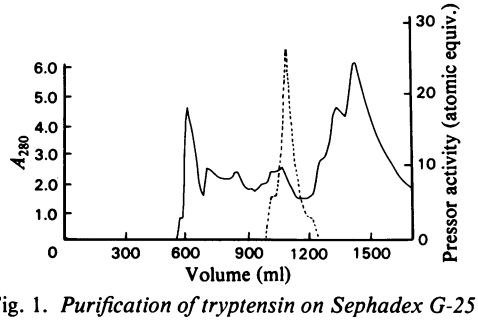

Fig. 1. *Purification of tryptensin on Sephadex G-25*
After adsorption on Dowex 50W (X2) the sample was purified on a Sephadex G-25 column (5cm × 109cm). Elution was with 0.1M-acetic acid containing 0.02% NaN_3 at a flow rate of 44ml/h, 15ml eluates being collected. The effluent was analysed by measurements of pressor activity (----; μg of synthetic angiotensin) and A_{280} (——).

Preparation of tryptensin. Dowex 50W (X2; NH_4^+ form) (150g) equilibrated with 0.2M-diethylamine acetate to pH6.0, was poured into a flask. To the flask was added 20g of fraction IV-4 dissolved in 800ml of phosphate-buffered saline [0.15M-$NaH_2PO_4 + Na_2HPO_4$/0.9% NaCl (1:1, v/v)], pH6.0, containing 0.02% NaN_3, and 800mg of 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK')-treated trypsin dissolved in 40ml of the above solution. To inhibit angiotensinase, 80ml of 0.1M-EDTA (ammonium salt), pH6.0, 8ml of 0.45M-8-hydroxyquinoline sulphate and 2ml of 2,3-dimercaptopropanol were added to the reaction mixture. The mixture was incubated at pH6.0 at 37°C for 3h. The Dowex 50W (X2) resin, which adsorbed the formed tryptensin, was separated from the remaining mixture on a glass filter column (5cm × 51cm). To the filtrate, another 15g of Dowex 50W (X2) resin was added and the mixture stirred gently for 1h at room temperature. Both resins were combined and washed with 7 litres of water on the glass filter until the washings became clear.

Purification of tryptensin. The Dowex resin remaining on the filter was eluted with 1.1 litres of 0.2M-diethylamine. The total pressor activity was equivalent to 245μg of synthetic [Ile^5]angiotensin and the specific activity was calculated to be 0.035.

Sephadex G-25 gel filtration. The eluate was evaporated to dryness under reduced pressure at a temperature of less than 45°C. It was then dissolved in 31ml of 0.1M-acetic acid containing 0.02% of NaN_3 and applied to a Sephadex G-25 column (5cm × 109cm). It was eluted with 0.1M-acetic acid containing 0.02% NaN_3 at a flow rate of 44ml/h and collected as 15ml fractions. The tubes con-

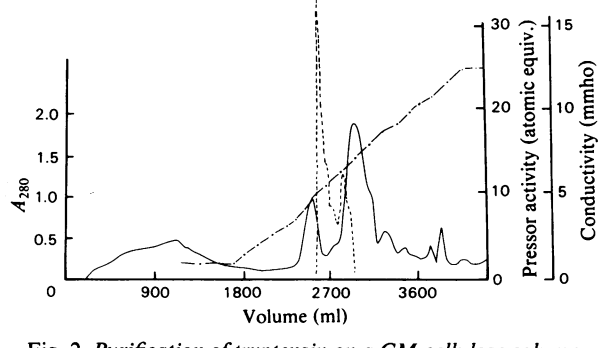

Fig. 2. *Purification of tryptensin on a CM-cellulose column*
After gel filtration on Sephadex G-25, the sample was chromatographed on a CM-cellulose column (3.7cm × 60cm) with gradient elution from 0.02M- to 0.15M-ammonium acetate buffer, pH5.2. Eluate was collected as 18ml fractions at a flow rate of 40ml/h. The effluent was analysed by measurements of pressor activity (----; μg of synthetic angiotensin) and A_{280} (——). -·-·-, Conductivity.

taining pressor activity were combined and subsequently freeze-dried. The pressor recovery was 97.8% and specific activity was 0.115 (Fig. 1).

CM-cellulose cation-exchange chromatography. The freeze-dried sample was dissolved in water. After being adjusted to the same pH and conductivity as 0.02M-ammonium acetate buffer, pH 5.2, it was chromatographed on a column (3.7cm × 60cm) of CM-52 CM-cellulose equilibrated with the same buffer. The elution was carried out at a flow rate of 40ml/h, beginning with 900ml of 0.02M-ammonium acetate buffer, and thereafter with a linear gradient elution from 1400ml of 0.02M- to 1400ml of 0.15M-ammonium acetate buffer. The elution was further continued with an additional 900ml of 0.15M-ammonium acetate buffer. It was collected as 18ml fractions. The effluent was analysed by measurements of pressor activity and A_{280}. The fractions with pressor activity were pooled. Recovery of pressor activity was 85.9% and specific activity was 1.183 (Fig. 2). The pooled fractions were desalted by means of the adsorption to Dowex 50W (X2) at room temperature. The resin was separated on a glass filter column (5cm × 51cm), washed with 2 litres of water and eluted with 750ml of 0.2M-diethylamine.

DEAE-cellulose anion-exchange chromatography. The eluate was evaporated to dryness under reduced pressure and dissolved in 300ml of water. The pH and conductivity were adjusted with the solvent pyridine/collidine/acetate buffer (pH 8.1; 1 litre of water, containing 10ml of pyridine and 10ml of collidine, was adjusted to pH 8.1 by adding acetic acid). This solution was applied to a column (3.8cm × 60cm) of DE-52 DEAE-cellulose, equilibrated with the same buffer. It was eluted first with 900ml of the same buffer and then with a linear gradient elution from 1400ml of pyridine/collidine/acetate buffer, pH 8.1, to 1400ml of pyridine/collidine/acetate buffer, pH 7.2. Fractions (20ml) were collected at a flow rate of 40ml/h. Of each fraction 0.2ml was used for Lowry's reaction and the A_{750} was read. Fractions with pressor activity were collected and freeze-dried. The yield of pressor activity was 68.8%, the specific activity being 11.3 (Fig. 3).

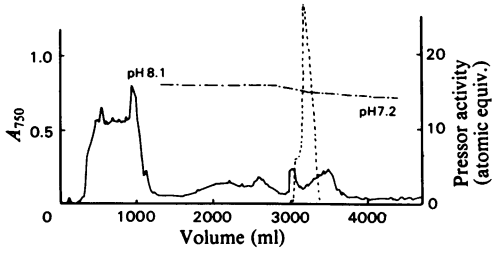

Fig. 3. *Purification of tryptensin on a DEAE-cellulose column (3.8cm × 60cm)*

Gradient elution was from pH 8.1 to 7.2 (pyridine/collidine/acetate buffer) at a flow rate of 40ml/h. Eluates were collected as 20ml fractions. The pressor activity (– – –; μg of synthetic angiotensin) and A_{750} (——) after Lowry's reaction were analysed. –·–·–, pH gradient.

CM-cellulose cation-exchange re-chromatography. The sample was dissolved in water and pH and conductivity were adjusted with 0.02M-ammonium acetate buffer, pH 5.2. This solution was applied to a column (2.8cm × 40cm) of CM-52 CM-cellulose equilibrated with the same buffer. Initially, it was eluted with 640ml of 0.02M-ammonium acetate buffer, pH 5.2, and thereafter with a linear gradient elution from 540ml of 0.02M-ammonium acetate buffer to 540ml of 0.15M-ammonium acetate buffer, pH 5.2, and finally with 400ml of 0.15M-ammonium acetate buffer. The eluate was collected in 8ml fractions at a flow rate of 25ml/h and the A_{280} was measured. The pressor-active fractions were separated from the major protein peak (Fig. 4). The recovery of pressor activity was 65.6% with a specific activity of 66.7. Fractions with pressor activity were combined and freeze-dried.

Bio-Gel P-2 gel filtration. The freeze-dried sample was dissolved in 1.5ml of 0.1M-acetic acid. This solution was applied to a column (1.5cm × 100cm) of Bio-Gel P-2. Elution was carried out with 0.1M-acetic acid, containing 0.02% NaN_3 at a flow rate of 7.5ml/h and 1.5ml fractions were collected. The pressor activity was recovered in 92.7% yield, with a specific activity of 452.8. The fractions with pressor activity were pooled and freeze-dried (Fig. 5).

High-pressure liquid chromatography. The sample was dissolved in 0.5ml of water, filtered through a 0.45μm Millipore filter, and applied to a Waters Associates model ALC/GPC 204 instrument of which the packing material was μ-Bondapak alkylphenyl and the column had the dimensions 4mm (internal diam.) × 30cm. The experiments were carried out at room temperature, solvents being filtered through 0.45μm Millipore filter and degassed. The A buffer was acetonitrile/0.1M-ammonium acetate mixture (3/17, v/v) and the B buffer was acetonitrile/0.1M-ammonium acetate mixture (9/1, v/v). The elution was with A buffer, to which 4% of B buffer was added, at a flow rate of 1.6ml/min. Each peak was bioassayed for pressor activity, and eluates comprising the peak with pressor activity were freeze-dried (Fig. 6).

Hydrolysis. A portion of the sample (25μg) was hydrolysed by 0.5ml of 6M-HCl in a sealed ampoule with N_2 gas at 110 ± 1°C for 20h. HCl was then

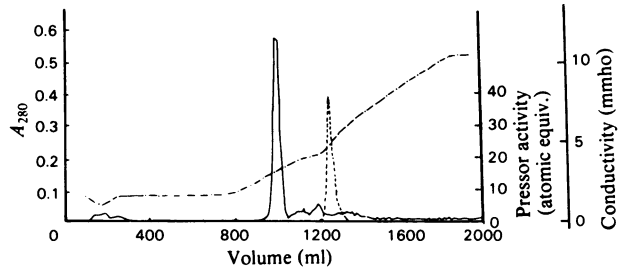

Fig. 4 *Purification of tryptensin on a CM-cellulose column*
After anion-exchange chromatography on DEAE-cellulose, the sample was re-chromatographed on a CM-cellulose column (2.8 cm × 40 cm) with gradient elution from 0.02 M- to 0.15 M-ammonium acetate buffer at pH 5.2. Fractions (8 ml) were collected at a flow rate of 25 ml/h. The effluent was analysed by measurements of pressor activity (----; μg of synthetic angiotensin) and A_{280} (——). -·-·-, Conductivity.

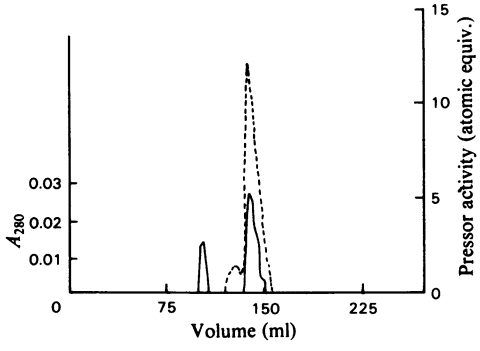

Fig. 5. *Purification of tryptensin on a Bio-Gel P-2*
After cation-exchange re-chromatography by CM-cellulose, the sample was chromatographed on a Bio-Gel P-2 column (1.5 cm × 100 cm). It was eluted in 0.1 M-acetic acid and collected in 1.5 ml fractions at a flow rate of 7.5 ml/h. The effluent was analysed by measurements of pressor activity (----; μg of synthetic angiotensin) and A_{280} (——).

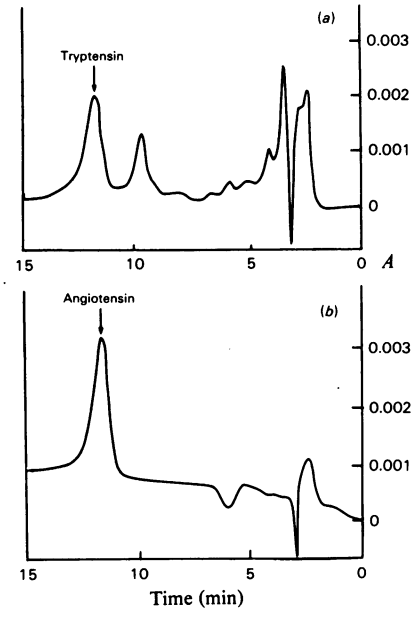

Fig. 6. *Isolation of tryptensin (a) and synthetic angiotensin (b) by high-pressure liquid chromatography*
The column material was μ-Bondapak alkylphenyl and the column dimensions were 4 mm internal diam. × 30 cm. Solvents: A buffer, containing 4% of B buffer [the composition of A buffer was acetonitrile/0.1 M-ammonium acetate mixture (3 : 17, v/v); the composition of B buffer was acetonitrile/0.1 M-ammonium acetate mixture (9 : 1, v/v)]. The flow rate was 1.6 ml/min and the detector measured u.v. absorbance at 254 nm. Synthetic angiotension was also chromatographed by high-pressure liquid chromatography. The conditions were the same as for tryptensin.

removed at 50°C under reduced pressure, and evaporated to dryness. The hydrolysed sample was analysed in a model 835 Hitachi high-speed amino acid analyser.

Results

The results for each step of the purification are summarized in Table 1. Gel filtration by Sephadex G-25 and cation-exchange chromatography on CM-cellulose facilitated removal of most of the inert

Table 1. *Recovery and specific activity in each step of the purification of tryptensin*

Recovery was calculated from the pressor activity. Specific activity was calculated from the ratio of pressor activity to the total absorbance determined at 280nm.

	Pressor activity (μg of synthetic [Ile^5]angiotensin)	Recovery of pressor activity (%)	A_{280}	Specific activity
1. Dowex 50W (X2)	245.0		7024	0.035
2. Sephadex G-25	239.5	97.8	2090	0.115
3. CM-cellulose	205.7	85.9	173.9	1.183
4. DEAE-cellulose	141.5	68.8	12.5	11.3
5. CM-cellulose (re-chromatography)	92.8	65.6	1.39	66.7
6. Bio-Gel P-2	86.0	92.7	0.190	452.8

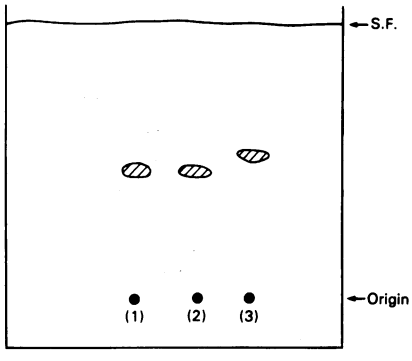

Fig. 7. *T.l.c. of isolated tryptensin and synthetic [Ile^5]-angiotensin and [Ile^5]proangiotensin.*

The compounds were chromatographed in isopentanol/pyridine/water (7:7:6, by vol.) on Avicel-SF cellulose thin-layer plates (10cm x 10cm) and developed with ninhydrin. (1) Synthetic [Ile^5]angiotensin; (2) isolated tryptensin; (3) synthetic [Ile^5]-proangiotensin. Abbreviation used: S.F., solvent front.

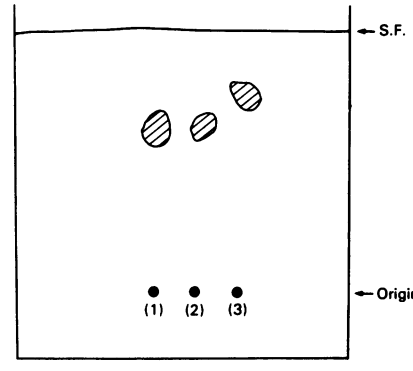

Fig. 8. *Thin-layer electrophoresis of isolated tryptensin and synthetic [Ile^5]angiotensin and synthetic [Ile^5]pro-angiotensin*

The compounds were subjected to electrophoresis on Avicil-SF cellulose thin-layer plates (10cm x 10cm) in pyridine/acetic acid/water (1:10:89, by vol.) at pH3.6 and 400V for 1h. The plate was developed with ninhydrin. (1) Synthetic [Ile^5]-angiotensin; (2) isolated tryptensin; (3) synthetic [Ile^5]-proangiotensin. Abbreviation used: S.F., solvent front.

proteins, purification being 34-fold. Next, the major protein of the residue was passed through the DEAE-cellulose resin and was separated from the pressor-active fractions by anion-exchange chromatography on DEAE-cellulose (Fig. 3). A 323-fold purification was achieved by this step, but minor contaminants could not be removed. To remove these, re-chromatography on CM-cellulose with gradient elution from 0.02M- to 0.15M-ammonium acetate buffer was performed; the pressor-active fraction being successfully separated from the protein peak (1906-fold purification). A further minor impurity was removed on Bio-Gel P-2, and the purification increased to 12937-fold (Fig. 5). For the final isolation, high-pressure liquid chromatography was used. By using the conditions described under 'Methods', the pure material gave the same elution profile as that obtained for synthetic human angiotensin (Fig. 6). The isolated tryptensin was chromatographed along with synthetic human pro-angiotensin and angiotensin by isopentanol/pyridine/water (7:7:6, by vol.) on an Avicel-SF cellulose thin-layer plate. Tryptensin showed the same R_F value (R_F0.47) as that of the synthetic angiotensin, but was apparently different from that of synthetic proangiotensin (R_F0.53), as shown in Fig. 7. The isolated tryptensin was subjected to electrophoresis on an Avicel-SF cellulose thin-layer plate in pyridine/acetic acid/water (1:10:89, by vol.) at pH3.6, applying 400V for 1h, along with synthetic human proangiotensin and angiotensin. Tryptensin showed the same mobility as that of synthetic [Ile^5]angiotensin as shown in Fig. 8. The

Table 2. *Amino acid composition of tryptensin compared with that of synthetic [Ile⁵]angiotensin*

The results are given as a molar ratio of each amino acid to isoleucine.

Amino acid	Tryptensin	Synthetic [Ile⁵]angiontensin
Asp	1.161	1.142
Val	1.228	1.188
Ile	1.000	1.000
Tyr	0.785	0.849
Phe	1.077	1.081
His	1.048	0.999
Arg	0.975	1.058
Pro	0.830	1.074

relative mobility of the isolated tryptensin compared with that of synthetic [Ile⁵]angiotensin was 1.0. Amino acid analysis of tryptensin hydrolysate was: Asp, 1.161; Val, 1.228; Ile, 1.0, Tyr, 0.785; Phe, 1.077; His, 1.048; Arg, 0.975; Pro, 0.830. The amino acid composition of tryptensin was identical with that of synthetic [Ile⁵]angiotensin (Table 2).

Discussion

Tryptensin had been isolated in a high degree of purity as evidenced by: (1) a single peak of pressor activity with A_{254} after the final partition chromatography on high-pressure liquid chromatography; (2) a single spot on t.l.c.; (3) also a single spot on electrophoresis.

Identity of tryptensin thus isolated with octapeptide angiotensin was supported by: (1) a similar R_F value and electrophoretic mobility as those of synthetic [Ile⁵]angiotensin respectively, which were apparently different from those of proangiotensin on t.l.c. and electrophoresis; (2) a similar amino acid composition to that of human angiotensin, namely Asp, Arg, Val, Tyr, Ile, His, Pro and Phe all in equimolar ratio. Proangiotensin, if present, would have been revealed by the present procedures, since these were based principally on the same procedures as those employed in the isolation of human proangiotensin (Arakawa *et al.*, 1967*a*,*b*). It must be emphasized that in that case there was found no angiotensin, indicating that there was no appreciable amount of converting enzyme in the reaction system. By contrast, in the present experiment, in which only renin was replaced by 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-treated trypsin, proangiotensin was not found at all in any step of the present purification procedures.

Trypsin is originally secreted into the intestinal tract *in situ* and digests food protein. But it can generate bradykinin in an experimental system *in vitro* (Elliott *et al.*, 1961). In fact, it was trypsin that was used to generate bradykinin, leading to the clarification of its chemical structure, with the result that trypsin has been known as a kinin-forming enzyme. In any case, the incubation with trypsin has been carried out naturally in a slightly alkaline pH environment. In contrast, we found that a slightly acidic pH, which is optimal for human renin, trypsin formed pressor-active substance from human plasma protein (Arakawa *et al.*, 1976). We had tentatively called it tryptensin, but it has now been shown to have the same amino acid composition as, and similar chromatographic profiles to, angiotensin. Although the amino acid sequence of tryptensin has not been elucidated, it is likely that it is identical with that of angiotensin.

Just how trypsin makes angiotensin is not known. Trypsin could activate a renin precursor, as has been reported by Morris & Lumbers (1972), Day & Leutscher (1975), Cooper *et al.* (1977), Sonenberg *et al.* (1977) and Sealey *et al.* (1979) in various conditions. However, it is not likely in the present experiment, since the conditions employed lack a sufficient amount of converting enzyme activity. Yet the product was not proangiotensin but angiotensin. Therefore, the direct formation of angiotensin is most likely. Whether, then, trypsin, having the capability of forming both pressor and depressor peptides, plays some role in regulating tissue blood perfusion and blood pressure in physiology remains to be elucidated.

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References

Arakawa, K., Nakatani, M. & Nakamura, M. (1965) *Nature (London)* **207**, 636

Arakawa, K., Nakatani, M. & Nakamura, M. (1967*a*) *Nature (London)* **214**, 278–279

Arakawa, K., Nakatani, M., Minohara, A. & Nakamura, M. (1967*b*) *Biochem. J.* **104**, 900–906

Arakawa, K., Minohara, A., Yamada, J., Uemura, N. & Nakamura, M. (1968) *Clin. Chim. Acta* **22**, 309–315

Arakawa, K., Minohara, A., Uemura, N., Sakai, T. & Ikeda, M. (1975) *Endocrinol. Jpn.* **22**, 427–432

Arakawa, K., Ikeda, M., Fukuyama, J. & Sakai, T. (1976) *J. Clin. Endocrinol. Metab.* **42**, 599–602

Cooper, R. M., Murray, G. E. & Osmond, D. H. (1977) *Circ. Res.* **40**, *Suppl.* **1**, 171–179

Day, R. P. & Leutscher, J. A. (1975) *J. Clin. Endocrinol. Metab.* **40**, 1085–1093

Elliott, D. F., Horton, E. W. & Lewis, G. P. (1961) *Biochem. J.* **78**, 60–65

Kostka, K. & Carpenter, F. H. (1964) *J. Biol. Chem.* **239**, 1799–1803

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Morris, B. J. & Lumbers, E. R. (1972) *Biochim. Biophys. Acta* **289**, 385–391

Sealey, J. E., Atlas, S. A., Laragh, J. H., Oza, N. B. & Ryan, J. W. (1979) *Hypertension* **1**, 179–189

Sonenberg, N., Wilchek, M. & Zamir, A. (1977) *Eur. J. Biochem.* **77**, 217–222